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(57) Abstract

The present invention comprises model systems that allow study and control of the activity of the intracellular or cytoplasmic regions of integrins and other transmembrane proteins. One aspect of these model systems is protein constructs that contain helical regions in order to approximate the effect of the transmembrane hydrophobic regions while maintaining solubility. One type of protein construct according to the present invention comprises: (1) a first segment including two amino acid sequences joined at their amino-terminal residues in head-to-head fashion, each of the two amino acid sequences of the first segment including a substantially helical amphiphilic amino acid region; and (2) two second segments, each of the second segments joined to the first segment at the carboxyl terminus of each of the two amino acid sequences of the first segment. The protein construct has one free alpha-amino terminus derived from one of the amino acid sequences of the first segment and two free carboxyl termini. Another type of protein construct according to the present invention comprises a first segment, two second segments, and two third segments. Also within the scope of the invention are chimeric integrins and methods for their use.

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STRUCTURAL MODELS FOR CYTOPLASMIC DOMAINS OF TRANSMEMBRANE RECEPTORS

BACKGROUND OF THE INVENTION

This invention is directed to structural models for cytoplasmic domains of transmembrane receptors, including protein constructs and chimeric proteins, as well as methods of synthesis and use of such structural models.

In eukaryotic cells, many proteins extend through the membrane and therefore have a cytoplasmic domain, a transmembrane domain, and an extracellular domain. Many of these proteins are involved in signal transduction, the control of cell adhesion, and cell-cell interaction control.

Among the proteins that fall into this category are the integrins (R.O. Hynes, "Integrins: Versatility, Modulation, and Signalling in Cell Adhesion," Cell 69:11-25 (1992); S.M. Albelda & C.A. Buck, "Integrins and Other Cell Adhesion Molecules," FASEB J. 4: 2868-2880 (1990); E. Ruoslahti, "Integrins," J. Clin. Invest. 87: 1-5 (1991); M.E. Hemler, "VLA Proteins in the Integrin Family: Structures, Functions, and Their Role on Leukocytes," Annu. Rev. Immunol. 8:365-400 (1990)).

Integrins are involved in a number of pathological and physiological processes, including thrombosis, inflammation, and cancer (M.J. Humphries et al., "A Synthetic Peptide From Fibronectin Inhibits Experimental Metastasis of Murine Melanoma Cells," Science 233:467-470 (1986); E. Ruoslahti, *supra*: 1-M.E. Hemler (1990), *supra*). Other physiological and pathological conditions involving changes in cell adhesiveness are also mediated through integrins.

Typically, transmembrane proteins are heterodimeric, being noncovalent associations of two or more different types of polypeptide subunits. In particular, integrins are heterodimers of two different protein subunits, designated α and β. The α subunits vary in size between 120 and 180 kDa and are each noncovalently associated

with a β subunit. The extracellular domain of the integrin molecule forms a ligand binding site; both the α and β subunits are involved in forming the ligand binding site. A number of different ligands for integrins are known are known, including collagens, laminin, fibronectin, vitronectin, complement components, thrombospondin, and integral membrane proteins of the immunoglobulin superfamily such as ICAM-1, ICAM-2, and VCAM-1. The integrins recognize various short peptide sequences in the ligands. Examples of these are Arg-Gly-Asp (RGD), Lys-Gln-Ala-Gly-Asp-Val (KQAGDV) (SEQ ID NO: 1), Asp-Gly-Glu-Ala (DGEA) (SEQ ID NO: 2), Glu-Ile-Leu-Asp-Val (EILDV) (SEQ ID NO: 3), and Gly-Pro-Arg-Pro (GPRP) (SEQ ID NO: 4). Variations in integrin function are often caused by changes in the ligand binding affinity of the extracellular domain of the integrins (J.S. Bennett & G. Vilaire, J. Clin. Invest. 64: 1393-1401 (1979); D.C. Altieri et al., J. Cell Biol. 107: 1893-1900 (1988); R.J. Faull et al., J. Cell Biol. 121: 155-162 (1993); B.A. Lollo et al., J. Biol. Chem. 268:21693-21700 (1993)).

Integrin $\alpha_{IIb}\beta_3$ (platelet GPIIb-IIIa), a heterodimer of two type I transmembrane protein subunits (R.O. Hynes (1992), *supra*), manifests highly regulated changes in ligand binding affinity. Affinity state-specific antibodies, e.g., PAC1 (S.J. Shattil et al., J. Biol. Chem. 260:1107-1114 (1985)), are useful for analysis of recombinant $\alpha_{IIb}\beta_3$ in heterologous cells (T.E. O'Toole et al., Cell Regulation 1:883-893 (1990)). Platelet agonists increase the affinity of $\alpha_{IIb}\beta_3$ (activation) probably by causing changes in the confirmation of the extracellular domain (T.E. O'Toole et al. (1990), *supra*; P.J. Sims et al., J. Biol. Chem. 266:7345-7352 (1991)). Cytoplasmic signaling pathways involving heterotrimeric GTP binding proteins, phospholipid metabolism, and serine-threonine kinases initiate these confirmational changes in the extracellular domain; these changes may also involve calcium fluxes, tyrosine kinases, and low

molecular weight GTP binding proteins (P.J. Sims et al. (1991), *supra*; S.J. Shattil et al., J. Biol. Chem. 267: 18424-18431 (1992); S.J. Shattil & J.S. Brugge, Curr. Opin. Cell Biol. 3: 869-879 (1991); M.H. Ginsberg et al., Cold Spring Harbor Symposium of Quantitative Biology: The Cell Surface 57: 221-231 (1992); M.H. Ginsberg et al., "Inside-Out Integrin Signalling," Curr. Opin. Cell Biol. 4: 766-771 (1992); Y. Nemoto et al., J. Biol. Chem. 267: 20916-20920 (1992)). How cytoplasmic signals result in changes in the conformation and ligand binding affinity of the extracellular domain ("inside-out signal transduction") of the integrin remains obscure. Studies with chimeras containing the cytoplasmic domains of various α and β subunits joined to the transmembrane and extracellular domain of $\alpha_{IIb}\beta_3$ indicates that integrin cytoplasmic domains transduce cell type-specific signals that modulate ligand binding affinity. These signals require active cellular processes in both α and β cytoplasmic tails of the integrin, suggesting that they reflect physiologically relevant signals. In addition, deletion of a highly conserved motif, Gly-Phe-Phe-Lys-Arg (GFFKR) (SEQ ID NO: 5), at the amino-terminus of the α subunit cytoplasmic domain, also resulted in high affinity binding of ligands to integrin $\alpha_{II}\beta_3$. In contrast to the chimeras, high affinity ligand binding to GFFKR (SEQ ID NO: 5) deletion mutants was independent of cellular metabolism, cell type, and the bulk of the β subunit cytoplasmic domain. Thus, integrin cytoplasmic tails are targets for the modulation of integrin affinity.

Although signal transduction by integrins, as well as by transmembrane proteins generally, is of great interest, technical difficulties have greatly limited the application of high resolution techniques for the structures of these proteins. Indeed, molecular structures are available for only two intact transmembrane proteins, a bacterial photoreaction center (J. Deisenhofer et al., Nature 318: 618-624 (1985)), and

a porin (M.S. Weiss et al., FEBS Lett. 267: 268-272 (1990)). Structures of receptor extracellular domains have been determined using soluble truncated extracellular domains as models (A.M. DeVos et al., Science 255: 306-312 (1992); M.V. Milburn et al., Science 254: 1342-1347 (1991)). These structures have contributed to the understanding of the basis of ligand recognition, but have provided less insight into the mechanism of signal transduction. Many membrane proteins that transduce signals are members of the Type I transmembrane protein family, the defining feature of which is a single membrane spanning region. These include the T cell receptor (A. Weiss, Cell 73: 209-212 (1993)); growth factor receptors (L. Patthy, Cell 61: 13-14 (1990), and cytokine receptors (A. Miyajima et al., TIBS 17: 378-382 (1992)). In general, the cytoplasmic domain of these proteins is critical for signaling. Thus, to understand signal transduction through such receptors, it is essential to understand the structure and function of the cytoplasmic domain. This is especially difficult for multisubunit Type I proteins.

An additional complication in studying and in developing methods of controlling or modulating the activity of integrins and other transmembrane proteins is the inherent hydrophobicity of membrane-spanning stretches of apolar amino acids. This means that structural studies on truncated receptors containing these helical regions will be impeded by insolubility in aqueous solution.

Therefore, there is a need for improved methods of controlling and modulating the activity of integrins and other transmembrane proteins, of detecting their activity, and modulating their activity to detect and control physiological conditions.

SUMMARY

To meet these needs, we have developed model systems that allow study and control of the activity of the intracellular or cytoplasmic regions of integrins and

other transmembrane proteins. Particularly, these model systems maintain the cytoplasmic regions in their natural conformation. These model systems include protein constructs and chimeric integrins.

One aspect of the present invention is protein constructs intended to link cytoplasmic regions of transmembrane proteins in a framework providing solubility in aqueous media. In general, a protein construct according to the present invention comprises:

(1) a first segment including two amino acid sequences joined at their amino-terminal residues in head-to-head fashion, each of the two amino acid sequences of the first segment including a substantially helical amphiphilic amino acid region; and

(2) two second segments, each of the second segments joined to the first segment at the carboxyl terminus of each of the two amino acid sequences of the first segment; the protein construct having: (i) either no free α-amino terminus or one free α-amino terminus derived from one of the two amino acid sequences of the first segment and (ii) two free carboxyl termini.

One protein construct according to the present invention comprises:

(1) a first segment including two amino acid sequences joined at their amino-terminal residues in head-to-head fashion, each of the two amino acid sequences of the first segment including a substantially helical amphiphilic amino acid region; and

(2) two second segments, each of the second segments joined to the first segment at the carboxyl terminus of each of the two amino acid sequences of the first segment.

The protein construct has one free α-amino terminus derived from one of the two amino acid sequences of the first segment and two free carboxyl termini. This is referred to as a Type I protein construct.

Typically, the substantially helical amphiphilic acid regions of the first segment have a predominantly

periodic secondary structure. Preferably, the substantially helical amphiphilic amino acid regions in the first segments have an estimated helicity of at least about 80%; more preferably, the helicity is at least about 85%.

Typically, the substantially helical amphiphilic amino acid regions each have the sequence G-$(X_1$-L-X_2-X_3-L-X_4-G$)_n$, wherein X_1 is selected from the group consisting of lysine, arginine, and ornithine, X_2 and X_4 are each independently selected from the group consisting of aspartic acid and glutamic acid, X_3 is selected from the group consisting of alanine, serine, and threonine, and n is an integer from 2 to 20. Preferably, n is from 3 to 6; more preferably, n is 4. Preferably, X_1 is lysine, X_2 and X_4 are each glutamic acid, and X_3 is alanine.

Typically, n is identical for both of the substantially helical amino acid regions; preferably, the sequence of both of the substantially helical amphiphilic amino acid regions is identical. However, these are not absolute requirements, and constructs in which n is different for the substantially helical amino acid regions or in which the sequence of the substantially helical amino acid regions differs are within the scope of the invention.

Typically, each of the two second segments has a length of about 15 amino acids to about 50 amino acids. The two second segments can be non-helical. Typically, the amino acid sequences of each of the two second segments are derived from the cytoplasmic domain of a transmembrane protein. More typically, the amino acid sequences of each of the two second segments are derived from the amino acid sequences of the cytoplasmic domains of the subunits of a heterodimeric multisubunit transmembrane protein where the subunits noncovalently associate *in vivo*. The amino acid sequences of each of the two second segments can be derived from the cytoplasmic domain of an integrin. In this case,

preferably the amino acid sequence of one of the second segments is derived from the cytoplasmic domain of an integrin selected from the group consisting of α_1, α_2, α_3, α_4, α_5, α_6, α_7, α_8, α_{IIb}, α_V, α_L, α_M, α_X, and α_{IEL}, and the amino acid sequence of the second of the second segments is derived from the cytoplasmic domain of an integrin selected from the group consisting of β_1, β_2, β_3, β_4, β_5, β_6, β_7, and β_8, so that one of the following combinations is formed: $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_4\beta_1$, $\alpha_4\beta_7$, $\alpha_5\beta_1$, $\alpha_6\beta_1$, $\alpha_6\beta_4$, $\alpha_7\beta_1$, $\alpha_8\beta_1$, $\alpha_V\beta_1$, $\alpha_V\beta_3$, $\alpha_V\beta_5$, $\alpha_V\beta_6$, $\alpha_V\beta_8$, $\alpha_L\beta_2$, $\alpha_M\beta_2$, $\alpha_X\beta_2$, $\alpha_{IIb}\beta_3$, and $\alpha_{IEL}\beta_7$.

A particularly preferred embodiment is one in which the amino acid sequence of one of the second segments is derived from the cytoplasmic domain of integrin α_{IIb} and the amino acid sequence of the second of the second segments is derived from the cytoplasmic domain of integrin β_3. In this embodiment, the amino acid sequence of the first of the second segments can be residues 989-1007 of integrin α_{IIb}, with an additional carboxyl-terminal glutamine, and the amino acid sequence of the second of the second segments can be that of residues 716-762 of integrin β_3.

Preferably, the helicity of at least one of the first and second segments is increased in the construct over the helicity of the first or second segment alone.

Typically, the two amino acid sequences of the first segment are linked by a thioether linkage between a cysteine residue at the amino terminus of one of the amino acid sequences of the first segment and a bromoacetyl moiety at the amino terminus of the second of the amino acid sequences of the first segment. Other linkages can also be used.

In an alternative Type I protein construct according to the present invention, the two amino acid sequences are linked by an oxime linkage between an aldehyde moiety and an aminooxy moiety. This alternative Type I protein construct can comprise:

(1) a first segment including two amino acid sequences joined at their amino-terminal residues in head-to-head fashion through an oxime linkage formed by reaction of an aldehyde moiety with an aminooxy moiety, each of the two amino acid sequences of the first segment including a substantially helical amphiphilic amino acid region; and

(2) two second segments, each of the second segments joined to the first segment at the carboxyl terminus of each of the two amino acid sequences of the first segment.

This alternative Type I protein construct has either zero or one free amino terminus derived from one of the two amino acid sequences of the first segment and two free carboxyl termini, with the proviso that when the protein construct has one free amino terminus, the amino acid residue having the aminooxy moiety has an amino group derived from a side chain, the free amino terminus of the protein construct being derived either from the α-amino terminus or from the amino terminus of the side chain.

One of the second segments can have an amino acid sequence derived from the cytoplasmic domain of α subunit of an integrin with a deletion of a sequence G-F-F-K-R.

A particularly preferred protein construct comprises:

(1) a first segment including two copies of a substantially helical amphiphilic amino acid sequence that is G-(K-L-E-A-L-E-G)$_4$, joined by a thioether linkage formed by reaction of a cysteine residue that is linked to the amino-terminal end of one of the two amino acid sequences and a bromoacetyl moiety that is linked to the amino-terminal end of the second amino acid sequence; and

(2) two second segments, each covalently linked to a carboxyl terminus of the first segment, wherein one of the second segments has the amino acid sequence of residues 989-1007 of integrin α_{IIb} with an additional carboxyl-terminal glutamine residue, and the second second segment is residues 716-762 of integrin β_3.

Another embodiment of the invention is a protein construct referred to herein as a Type II protein construct, which comprises:

(1) a first segment including two amino acid sequences joined at their amino-terminal residues in head-to-head fashion so that the first segment has no free amino terminus and two carboxyl termini available for covalent linkage to the amino-terminal end of another amino acid segment;

(2) two second segments, each including a substantially helical amphiphilic amino acid region, each of the second segments being covalently linked at their amino-terminal ends to a carboxyl terminus of the first segment; and

(3) two third segments, each of the third segments being an amino acid sequence, each of the third segments being covalently linked by their amino-termini to the carboxyl-termini of the second segment, the protein construct having no free amino terminus and two free carboxyl termini derived from the third segments.

The second and third segments of this Type II protein construct are analogous to the first and second segments of the Type I protein construct, but the Type II protein construct has additional residues between the helical regions and the covalent linkage. The first segment can include a specific binding partner sequence having affinity for a specific binding partner. The specific binding partner sequence can specifically bind an antibody.

Another aspect of the present invention is a method of producing protein constructs. For Type I protein constructs using a thioether linkage, this method can comprise the steps of:

(1) providing two amino acid sequences, each amino acid sequence including: (i) an amino-terminal amino acid segment; (ii) an intermediately situated substantially helical amphiphilic amino acid segment covalently linked at its amino terminus to the first segment; and (iii) a

third amino acid segment covalently linked at its amino terminus to the second amino acid segment within the amino acid sequence; and

(2) joining the two amino acid sequences in head-to-head fashion through a thioether linkage to form a protein construct having one free α-amino terminus derived from one of the two amino acid sequences and two free carboxyl termini.

For Type I protein constructs using an alternative oxime linkage, the method can comprise the steps of:

(1) providing two amino acid sequences, each amino acid sequence comprising a substantially helical amphiphilic amino acid region at its amino terminus covalently linked to a second amino acid sequence; and

(2) joining the two amino acid sequences in head-to-head fashion through an oxime linkage between an amino acid residue with an aldehyde moiety and an amino acid residue with an aminooxy moiety to form a protein construct having either zero or one free amino terminus derived from one of the two amino acid sequences of the first segment and two free carboxyl termini, with the proviso that when the protein construct has one free amino terminus, the amino acid residue having the aminooxy moiety has an amino group derived from a side chain, the free amino terminus of the protein construct being derived either from the α-amino terminus or from the amino terminus of the side chain.

For Type II protein constructs using a thioether linkage, the method can comprise the steps of:

(1) providing two amino acid sequences, each amino acid sequence including: (i) an amino-terminal amino acid segment; (ii) an intermediately situated substantially helical amphiphilic amino acid segment covalently linked at its amino terminus to the first segment; and (iii) a third amino acid segment covalently linked at its amino terminus to the second amino acid segment within the amino acid sequence; and

(2) joining the two amino acid sequences in head-to-

head fashion through an oxime linkage between an amino acid residue with an aldehyde moiety and an amino acid residue with an aminooxy moiety to form a protein construct having either zero or one free amino terminus derived from one of the two amino acid sequences of the first segment and two free carboxyl termini, with the proviso that when the protein construct has one free amino terminus, the amino acid residue having the aminooxy moiety has an amino group derived from a side chain, the free amino terminus of the protein construct being derived either from the α-amino terminus or from the amino terminus of the side chain.

For alternative Type II protein constructs using an oxime linkage, the method can comprise the steps of:

(1) providing two amino acid sequences, each amino acid sequence including: (i) an amino-terminal amino acid segment; (ii) an intermediately situated substantially helical amphiphilic amino acid segment covalently linked at its amino terminus to the first segment; and (iii) a third amino acid segment covalently linked at its amino terminus to the second amino acid segment within the amino acid sequence; and

(2) joining the two amino acid sequences in head-to-head fashion through an oxime linkage between an amino acid residue with an aldehyde moiety and an amino acid residue with an aminooxy moiety to form a protein construct having either zero or one free amino terminus derived from one of the two amino acid sequences of the first segment and two free carboxyl termini, with the proviso that when the protein construct has one free amino terminus, the amino acid residue having the aminooxy moiety has an amino group derived from a side chain, the free amino terminus of the protein construct being derived either from the α-amino terminus or from the amino terminus of the side chain.

Another aspect of the present invention is chimeric

integrin proteins and methods for their use.

Chimeric integrin proteins according to the present invention can comprise: (1) a chimeric integrin protein comprising the extracellular and transmembrane domains of the Tac subunit of the IL-2 receptor covalently linked to the cytoplasmic domain of integrin β_3; (2) a chimeric integrin protein comprising the extracellular and transmembrane domains of the Tac subunit of the IL-2 receptor covalently linked to the cytoplasmic domain of integrin β_3 with amino acid 752 being mutated from a serine residue to a proline residue; (3) a chimeric integrin protein comprising the transmembrane and extracellular domains of the Tac subunit of the human IL-2 receptor covalently linked to the cytoplasmic domain of integrin α_{IIb}; and (4) a heterodimeric chimeric integrin in which the extracellular and transmembrane domains of human integrin $\alpha_{IIb}\beta_3$ are joined to the cytoplasmic domains of human integrin $\alpha_5\beta_1$.

Another aspect of the invention is nucleic acid sequences encoding the chimeric integrin proteins. Yet another aspect of the invention is these nucleic acid sequences operatively linked to at least one control element for transcription of the nucleic acid sequence, as well as vectors comprising the nucleic acid sequences operatively linked to the control elements, the vectors being capable of transfecting at least one eukaryotic host for expression of the chimeric integrin proteins encoded by the nucleic acid sequences.

Another aspect of the invention is a method for blocking the activation of a human cellular integrin comprising the step of expressing a chimeric integrin whose extracellular and transmembrane domains are derived from the Tac subunit of the human IL-2 receptor and whose cytoplasmic domain is the cytoplasmic domain of the human β_1 integrin in a quantity sufficient to inhibit high affinity ligand binding by the cellular integrin. Alternatively, in this method, the chimeric integrin

protein comprising the extracellular and transmembrane domains of the Tac subunit of the IL-2 receptor covalently linked to the cytoplasmic domain of integrin β_3 can be used.

BRIEF DESCRIPTION OF THE DRAWINGS

These and other features, aspects, and advantages of the present invention will become better understood with reference to the following description, appended claims, and the accompanying drawings where:

Figure 1A is a schematic drawing of the presumed topology of the native integrin $\alpha_{IIb}\beta_3$;

Figure 1B is a schematic drawing of a Type I protein construct of the present invention designated MP-1;

Figure 2 is a depiction of the amino acid sequences of the synthetic polypeptide targets, including a helix-dimer, MP-1, and MP-2, showing the relationships between the sequences in the assembled molecules;

Figure 3 is a schematic equation showing the synthesis of MP-1 using chemical dovetailing;

Figure 4 shows graphs of analytical HPLC results illustrating the effect of reaction conditions on formation of the thioether bond in the chemical dovetailing step as a function of time: (4(a), aqueous solution (0.1 M phosphate, pH 7); 4(b), dimethylformamide-0.1 M phosphate (95:5), pH 7);

Figure 5 shows the results of studies intended to characterize the assembled polypeptides MP-1 (5(a)) and MP-2 (5(b)); main panel, ion-spray mass spectra showing charge state distribution; insets, analytical reverse phase HPLC spectra;

Figure 6 shows the results of circular dichroism studies of synthetic polypeptides containing the individual cytoplasmic domains of the integrin $\alpha_{IIb}\beta_3$ (6(a), isolated cytoplasmic domains, 6(b), cytoplasmic domains attached to helical segments);

Figure 7 shows the results of circular dichroism studies of MP-1, MP-2, and the covalent helix-dimer;

Figure 8 shows the results of fluorescence quenching

studies on MP-1, MP-2, isolated β_3, and isolated β_3 + isolated α_{IIb};

Figure 9 is a schematic depiction of various chimeric integrins and Tac chimeras, including the amino acid sequences of the cytoplasmic regions of the integrins;

Figure 10 shows histograms depicting the results of flow cytometry in which fluorescence intensity is depicted on the abscissa and cell number on the ordinate; in Panels A, B, and C, PAC1 binding in the absence (filled histogram) or presence (open histogram) of competitive inhibitor is shown; in Panel D, surface expression of $\alpha_{IIb}\beta_3$ is shown as reported by the binding of D57 antibody in the absence (filled histogram) or presence (open histogram) of co-transfected Tac-β_3 DNA; M1 indicates the region containing those cells that express the recombinant $\alpha_{IIb}\beta_3$ construct bearing the cytoplasmic domains of β_1 and α_5;

Figure 11A is a graph showing the inhibition of inside-out signaling by Tac chimeras ((■) β_3, (●) β_1, (▲) α_5) used to transfect CHO cells, with signaling assessed by PAC1 binding;

Figure 11B is a graph showing the expression of the Tac chimeras used in Figure 11A assessed by the binding of the anti-TAC antibody 7G7B6;

Figure 12A is a graph showing the effect of the mutation $S^{752}P$ on inside-out signaling in cells transfected with chimeras as in Figure 11A ((▲) Tac-β_3($S^{752}P$); (■) Tac-β_3);

Figure 12B is a graph showing the expression of the Tac chimeras used in Figure 11A assessed as in the experiments shown in Figure 11A and 11B;

Figure 13A is a graph showing the effect of a Tac-β_1 chimera on the affinity of a "hinge" mutant, $\alpha_L\Delta$ ((●) α_5; (■) $\alpha_L\Delta$), by cotransfection as in Figure 11A; and

Figure 13B is a graph showing the expression of the Tac-β_1 chimera in the experiments of Figure 13A assessed

as in the experiments shown in Figure 11A and 11B.

DESCRIPTION

Definitions

In the context of this disclosure, including the description, examples, and claims, the following terms are defined as follows unless otherwise indicated:

"Protein Construct" means a molecule constructed from two or more protein segments that are covalently joined in such a way that the molecule has one free α-amino terminus derived from one of the protein segments but two or more free carboxyl termini.

"Nucleic Acid" includes both DNA and RNA unless otherwise indicated, and can include both single and double-stranded nucleic acid sequences. If a DNA sequence is referred to, references generally to both strands of a DNA sequence, either individually or as a Watson-Crick double helix. If only one strand is specified, the complementary strand whose antiparallel sequence is determined by Watson-Crick based pairing rules is also included unless the complementary sequence is specifically excluded. If only one strand is specified in double-stranded DNA, the strand specified as the sense strand whose strand would be equivalent to the sequence of any RNA transcribed from the double-stranded DNA, except for the replacement of thymidine (T) in the DNA by uridine (U) in the RNA. Reference to a nucleic acid sequence also includes modified bases as long as the modification does not significantly interfere with Watson-Crick base pairing or other specified functions of the nucleic acid, and can, for example, include substitution of uridine for thymidine in DNA as well as methylation of bases or modification of sugars.

"Helical" refers to any periodic regular secondary structure of amino acids in a protein, domain of a protein, or peptide characterized by a uniform translation along an axis, and includes but is not limited to an α-helix unless so specified.

"Periodic" refers to any regular secondary structure

of amino acids in a protein that is not a random coil or a quasi-random coil, and includes, but is not limited to, a helical structure.

<u>"Specific binding partner"</u> refers to a member of a pair of molecules that interact by means of specific non-covalent interactions that depend on the three-dimensional structures of the molecules involved, such as salt links, hydrophobic interactions, and van der Waals interactions. Typical pairs of specific binding partners include antigen-antibody, hapten-antibody, hormone-receptor, nucleic acid strand-complementary nucleic acid strand, substrate-enzyme, inhibitor-enzyme, carbohydrate-lectin, biotin-avidin, and virus-cellular receptor.

<u>"Antibody"</u> includes both intact antibodies and antibody fragments possessing an antigen-binding site, as well as chimeric antibodies.

<u>"Amino terminus"</u> refers to an amino group or moiety that is part of an amino acid residue within a protein, polypeptide, or protein construct. The amino group can either be an α-amino terminus or an amino terminus from a side chain, such as the ϵ-amino group of lysine.

<u>"Amino-terminal residue"</u> refers to an entire amino acid residue possessing an amino terminus. The amino-terminal residue can be joined to other amino acid residues, either through its carboxyl terminus by a peptide bond, or through a side chain.

I. <u>PROTEIN CONSTRUCTS</u>

One aspect of the present invention is protein constructs that maintain the cytoplasmic tails of transmembrane proteins in conformations approaching their naturally occurring conformations while preserving solubility in an aqueous medium and obviating the difficulty of working with proteins containing highly hydrophobic transmembrane segments.

These protein constructs allow study of the conformation and activity of the cytoplasmic regions of transmembrane proteins, including integrins, and can be used, as discussed below, in screening and in controlling

the activity of integrins and other transmembrane proteins.

A. Structure of Protein Constructs

In general, a protein construct according to the present invention comprises:

(1) a first segment including two amino acid sequences joined at their amino-terminal residues in head-to-head fashion, each of the two amino acid sequences of the first segment including a substantially helical amphiphilic amino acid region; and

(2) two second segments, each of the second segments joined to the first segment at the carboxyl terminus of each of the two amino acid sequences of the first segment;

the protein construct having: (i) either no free α-amino terminus or one free α-amino terminus derived from one of the two amino acid sequences of the first segment and (ii) two free carboxyl termini.

1. Protein Construct With First and Second Segments

One aspect of the present invention is a protein construct with first and second segments. In general, this protein construct comprises:

(1) a first segment including two amino acid sequences joined at their amino-terminal residues in head-to-head fashion, each of the two amino acid sequences of the first segment including a substantially helical amphiphilic amino acid region; and

(2) two second segments, each of the second segments joined to the first segment at the carboxyl terminus of each of the two amino acid sequences of the first segment. The protein construct has one free α-amino terminus derived from one of the two amino acid sequences of the first segment and two free carboxyl termini. For convenience, this protein construct is referred to herein as a Type I protein construct.

The free α-amino terminus derived from one of the two amino acid sequences of the first segment is free in

the sense that it is not involved in a peptide bond. However, unlike the situation in naturally-occurring proteins, the amino acid residue to which this α-amino terminus belongs is covalently linked to two other amino acid residues, although one of the linkages is through a side chain; the other is through the carboxyl terminus of that residue, through a peptide bond. This α-amino terminus derived from one of the amino acid sequences of the first segment must be distinguished from any amino terminus derived from the side chains of the amino acid sequence, typically ε-amino termini derived from lysine side chains.

Typically, the substantially helical amphiphilic acid regions of the first segment have a predominantly periodic secondary structure. More typically, the substantially helical amphiphilic amino acid regions in the first segment have an estimated helicity of at least about 80%. Preferably, the substantially helical amphiphilic amino acid regions in the first segment have an estimated helicity of at least about 85%. One suitable measurement technique for helicity is circular dichroism (CD), but other measurement techniques such as optical rotatory dispersion (ORD) can be used.

Typically, the substantially helical amphiphilic amino acid regions have the sequence is $G\text{-}(X_1\text{-}L\text{-}X_2\text{-}X_3\text{-}L\text{-}X_4\text{-}G)_n$. In this repeating sequence, n is an integer from 2 to 20, preferably from 3 to 6, more preferably 4. In the repeating sequence, X_1 is lysine, arginine or ornithine, X_2 and X_4 can each be glutamic acid or aspartic acid, and X_3 can be alanine, serine, or threonine. Typically, the sequence of the two substantially helical amphiphilic amino acid regions is the same, but this is not required. Typically, n is the same for both of the two substantially helical amphiphilic amino acid regions, but this is also not required. In some applications, it may be desirable to provide protein constructs in which the substantially helical amphiphilic regions are of different lengths.

Preferably, X_1 is lysine, X_2 and X_4 are each glutamic acid, and X_3 is alanine. Derivatives of these amino acids that do not alter the relative hydrophobicity or hydrophilicity or disrupt the helix are also included within the scope of the invention.

In one particularly preferred alternative of the invention, the helical amphiphilic amino acid regions form coiled-coil tertiary and quaternary structures (O.D. Monera et al., "Comparison of Antiparallel and Parallel Two-Stranded α-Helical Coiled-Coils," J. Biol. Chem. 268: 19218-19227 (1993); N.E. Zhou et al., J. Biol. Chem. 267: 2664-2670 (1992)) analogous to those of the prototypical coiled-coil protein tropomyosin. The stability of these coiled-coils is largely a result of strong interchain hydrophobic interactions between leucine residues in the seven residue repeat (S.Y.M. Lau et al., J. Biol. Chem. 259: 23253-13261 (1984)). These coiled-coil structures are likely to better mimic the proximity of transmembrane helices in the natural system and also ensure that a defined topology is maintained between the α and β cytoplasmic tails. In other words, the coiled-coil can act as a structural template onto which the cytoplasmic domain of the integrin or other transmembrane protein is attached. This ensures that the two cytoplasmic tails are staggered with respect to one another in a manner that approximates the intact protein.

Typically, each of the two second segments has a length of about 10 amino acids to about 80 amino acids; more typically, each of the two second segments has a length of about 15 amino acids to about 50 amino acids. The two second segments can be predominantly non-helical in structure. The amino acid sequences of each of the two second segments can be derived from the cytoplasmic domain of a transmembrane protein. Typically, the amino acid sequences of the two second segments are derived from the amino acid sequences of the cytoplasmic domains of the subunits of a heterodimeric multisubunit transmembrane protein where the subunits noncovalently

associate *in vivo*, but it is also possible to prepare constructs according to the present invention in which the amino acid sequences of the second segments are derived from protein subunits that do not normally associate into a heterodimeric transmembrane protein *in vitro*.

In one embodiment, the amino acid segments of each of the two second segments are derived from the cytoplasmic domain of an integrin. The protein constructs of the present invention are particularly useful for mimicking integrin conformation because, in a heterodimeric integrin, the location of the transmembrane-cytoplasmic interface of both families of subunits is formed by conserved Trp-Lys or Tyr-Lys residues. These residues are proximal to each other at the interface, so that subunits of differing lengths have a predictable relative stagger. The cytoplasmic tails are connected to helical transmembrane stretches, which may be important for inducing structure along the tails, and both cytoplasmic tails have carboxyl termini.

The following combinations of sequences from the α and β subunits of integrins can be used according to Hynes (1992), *supra*, incorporated herein in its entirety by this reference: Subunit β_1 can associate with any of α_1, α_2, α_3, α_4, α_5, α_6, α_7, α_8, and α_V. The amino acid sequence from β_2 can be associated with the amino acid sequence from α_L, α_M and α_X. The amino acid sequence from β_3 can be associated with α_{IIb} or α_V. The amino acid sequence of subunit β_4 can be associated with α_6. For subunits β_5, β_6, and β_8, the amino acid sequence can be associated with the amino acid sequence of α_V. For β_7, the amino acid sequence can be associated with the amino acid sequence for α_4 or α_{IEL}. The term "associated" in this context means that a heterodimeric integrin in which the cytoplasmic domains of the sequences are as specified is likely to form *in vivo*.

In one particularly preferred embodiment, the amino

acid sequence of one of the second segments is derived from the cytoplasmic domain of integrin α_{IIb} and the amino acid sequence of the other of the second segments is derived from the cytoplasmic domain of β_3.

Preferably, the amino acid sequence of the first of the second segments is derived from residues 989-1008 of α_{IIb} and the amino acid sequence of the second of the second segments is that of residue 716-762 of β_3. The residue 1008, which is glutamic acid (E) in the natural integrin according to the DNA sequence, is preferably altered to glutamine (Q) in the protein construct, because it is believed that post-translational side-chain amidation of the carboxyl-terminal glutamate occurs (J.J. Calvete et al., <u>FEBS Lett.</u> 263:43-46 (1990)).

Although one particularly useful protein construct derives its second segments from sequences of integrins, the sequences of other transmembrane proteins can be used. These include the T-cell receptor, cytokine receptor, other CD antigens, or the growth hormone receptor.

The two amino acid sequences of the first segment are joined at their amino-terminal residues in head-to-head fashion so that there is one free α-amino terminus, as described above. Preferably, the first and second segments are joined through a thioether linkage. Other joining methods also are known in the art.

In an alternative Type I protein construct according to the present invention, the first and second segments are joined through an oxime linkage formed between an aminooxy moiety on the amino-terminal residue of one of the two amino acid sequences of the first segment and an aldehyde residue on the amino-terminal residue of the other amino acid sequence of the first segment. This protein construct comprises:

(1) a first segment including two amino acid sequences joined at their amino-terminal residues in head-to-head fashion through an oxime linkage formed by reaction of an aldehyde moiety with an aminooxy moiety,

each of the two amino acid sequences of the first segment including a substantially helical amphiphilic amino acid region; and

(2) two second segments, each of the second segments joined to the first segment at the carboxyl terminus of each of the two amino acid sequences of the first segment.

The protein construct has either zero or one free amino terminus derived from one of the two amino acid sequences of the first segment and two free carboxyl termini, with the proviso that when the protein construct has one free amino terminus, the amino acid residue having the aminooxy moiety has an amino group derived from a side chain, the free amino terminus of the protein construct being derived either from the α-amino terminus or from the amino terminus of the side chain.

In this alternative Type I protein construct, it is not necessary that the linkage of the two amino acid sequences of the first segment be through the side chain of a cysteine residue. If a free α-amino terminus exists in this alternative Type I protein construct, it is available for reaction.

Typically, in the constructs of the present invention, the helicity of at least one of the first and second segments is increased in the construct over the helicity of the first or second segment alone, as measured by circular dichroism.

In another embodiment of the present invention, one of the second segments has an amino acid sequence derived from the α subunit of an integrin, such as α_{IIb}, with a deletion of the sequence GFFKR (SEQ ID NO: 5). This deletion causes an increase of the affinity of the integrin for its extracellular ligand and is sometimes referred to as a "hinge mutation." A schematic of a typical Type I protein construct of the present invention, using second segments whose amino acid sequences are derived from α_{IIb} and β_3 integrins, is shown in Figure 1.

Although the protein constructs disclosed above contain two cytoplasmic domains of transmembrane proteins, related techniques of chemoselective ligation can be used to generate higher-order protein constructs containing three, four, or more cytoplasmic domains of transmembrane proteins held in a conformation approximating the conformation *in vivo*. This technique is known generally as template-assisted protein synthesis, and is described in P.E. Dawson & S.B.H. Kent, "Convenient Total Synthesis of a 4-Helix TASP Molecule by Chemoselective Ligation," J. Am. Chem. Soc. 115: 7263-7266 (1993).

2. Protein Construct With First, Second, and Third Segments

Another embodiment of the present invention is a protein construct comprising:

(1) a first segment including two amino acid sequences joined at their amino-terminal residues in head-to-head fashion so that the first segment has one free α-amino terminus derived from one of the two amino acid sequences of the first segment and two carboxyl termini available for covalent linkage to the amino-terminal end of another amino acid segment;

(2) two second segments, each including a substantially helical amphiphilic amino acid region, each of the second segments being covalently linked at their amino-terminal ends to a carboxyl terminus of the first segment; and

(3) two third segments, each of the third segments being an amino acid sequence, each of the third segments being covalently linked by their amino-termini to the carboxyl-termini of the second segment. The protein construct has one free amino terminus derived from the first segment and two free carboxyl termini derived from the third segments. For convenience, this construct is referred to as a Type II protein construct herein.

The second and third segments of the Type II protein construct are substantially equivalent to the first and

second segments of the Type I protein construct described above. However, the first segment includes additional amino acid residues.

The first segment of the Type II construct can include a specific binding partner sequence having affinity for a specific binding partner. The specific binding partner sequence can specifically bind an antibody.

An alternative Type II protein construct uses an oxime linkage between two amino acid sequences of the first segment as discussed above. This protein construct comprises:

(1) a first segment including two amino acid sequences joined at their amino-terminal residues in head-to-head fashion through an oxime linkage formed by reaction of an aldehyde moiety with an aminooxy moiety so that the first segment has two carboxyl termini available for covalent linkage to the amino-terminal end of another amino acid segment;

(2) two second segments, each including a substantially helical amphiphilic amino acid region, each of the second segments being covalently linked at their amino-terminal ends to a carboxyl terminus of the first segment; and

(3) two third segments, each of the third segments being an amino acid sequence, each of the third segments being covalently linked by their amino-termini to the carboxyl-termini of the second segment.

This alternative Type II protein construct has either zero or one free amino terminus derived from one of the two amino acid sequences of the first segment and two free carboxyl termini, with the proviso that when the protein construct has one free amino terminus, the amino acid residue having the aminooxy moiety has an amino group derived from a side chain, the free amino terminus of the protein construct being derived either from the α-amino terminus or from the amino terminus of the side chain.

B. Synthesis of Protein Constructs

Typically, synthesis of a type I protein construct using a thioether linkage according to the present invention occurs by the following process:

(1) providing two amino acid sequences, each amino acid sequence comprising a substantially helical amphiphilic amino acid region at its amino terminus covalently linked to a second amino acid sequence; and

(2) covalently linking two amino acid sequences in head-to-head fashion through a thioether linkage to produce a protein construct having one free α-amino terminus derived from one of the two amino acid sequences and two free carboxyl termini.

Typically, synthesis of an alternative Type I protein construct using an aminooxy linkage occurs by the following process:

(1) providing two amino acid sequences, each amino acid sequence comprising a substantially helical amphiphilic amino acid region at its amino terminus covalently linked to a second amino acid sequence; and

(2) joining the two amino acid sequences in head-to-head fashion through an oxime linkage between an amino acid residue with an aldehyde moiety and an amino acid residue with an aminooxy moiety to form a protein construct having either zero or one free amino terminus derived from one of the two amino acid sequences of the first segment and two free carboxyl termini, with the proviso that when the protein construct has one free amino terminus, the amino acid residue having the aminooxy moiety has an amino group derived from a side chain, the free amino terminus of the protein construct being derived either from the α-amino terminus or from the amino terminus of the side chain.

Typically, synthesis of a Type II protein construct using a thioether linkage according to the present invention occurs by the following process:

(1) providing two amino acid sequences, each amino acid sequence including: (i) an amino-terminal amino acid

segment; (ii) an intermediately situated substantially helical amphiphilic amino acid segment covalently linked at its amino terminus to the first segment; and (iii) a third amino acid segment covalently linked at its amino terminus to the second amino acid segment within the amino acid sequence; and

(2) joining the two amino acid sequences in head-to-head fashion through a thioether linkage to form a protein construct having one free α-amino terminus derived from one of the two amino acid sequences and two free carboxyl termini.

Typically, synthesis of an alternative Type II protein construct using an aminooxy linkage occurs by the following process:

(1) providing two amino acid sequences, each amino acid sequence including: (i) an amino-terminal amino acid segment; (ii) an intermediately situated substantially helical amphiphilic amino acid segment covalently linked at its amino terminus to the first segment; and (iii) a third amino acid segment covalently linked at its amino terminus to the second amino acid segment within the amino acid sequence; and

(2) joining the two amino acid sequences in head-to-head fashion through an oxime linkage between an amino acid residue with an aldehyde moiety and an amino acid residue with an aminooxy moiety to form a protein construct having either zero or one free amino terminus derived from one of the two amino acid sequences of the first segment and two free carboxyl termini, with the proviso that when the protein construct has one free amino terminus, the amino acid residue having the aminooxy moiety has an amino group derived from a side chain, the free amino terminus of the protein construct being derived either from the α-amino terminus or from the amino terminus of the side chain.

The amino acid sequences comprising the first and second segments or the first, second, and third segments are generally synthesized through peptide synthesis. The

principles of peptide synthesis, particularly solid-phase peptide synthesis, are well known in the art and are described, for example, in M. Bodanszky, "Principles of Peptide Synthesis" (2d ed., Springer, Berlin, 1993). Typically, synthesis is performed on a solid phase, and involves activation and coupling, the reversible blocking of amino and carboxyl groups, and eventual deprotection. A particularly useful method of solid phase protein synthesis is described in M. Schnölzer, "In Situ Neutralization in Boc-Chemistry Solid Phase Peptide Synthesis," Int. J. Peptide Protein Res. 40:180-193 (1992), incorporated herein by this reference. This method uses a t-butyloxycarbonyl protecting group.

Alternatively, the amino acid sequences needed for assembly of the constructs can be produced through recombinant expression techniques that yield the desired products. These techniques are well known in the art and need not be described further here.

The synthesized segments are then covalently linked in head-to-head fashion. Preferably, a free cysteine residue on one of the amino acid sequences is condensed with a bromoacetyl moiety on the other amino acid sequence to yield a protein construct with a thioether linkage. Such techniques are described, for example in, T.W. Muir & S.B.H. Kent, "The Chemical Synthesis of Proteins," Curr. Opin. Biotechnology 4:420-427 (1993) and in M. Schnölzer & S.B.H. Kent, Science 256:221-225 (1992), both of which are incorporated herein by this reference.

C. Applications

The protein constructs of the present invention have a number of applications based on the ability to maintain the cytoplasmic tails of the construct in a configuration that is equivalent or similar to the configuration predominating *in vivo* while maintaining solubility and stability in an aqueous system, namely in staggered, parallel, and proximal topology. For example, these protein constructs can be used to detect intracellular

molecules capable of binding to integrins and modulating signals by inside-out signaling. Alternatively, these molecules can be used *in vivo* to disrupt or modulate inside-out signaling by binding to the cells in a manner such that the cytoplasmic domains of these protein constructs compete for intracellular molecules with the natural integrins. Because these protein constructs do not contain the extracellular ligand-binding sites of integrins, they would then disrupt inside-out signaling. This would be particularly useful in conditions in which overactivity of integrins is involved, such as inflammation, thrombosis, and malignancy.

Additionally, protein constructs according to the present invention could be used to detect molecules capable of binding to the intracellular or cytoplasmic domain of integrins and other transmembrane molecules *in vivo*. The protein constructs could be modified with aldehyde or aminooxy groups and cross-linked to affinity columns for chromatography. Affinity chromatography is well known in the art and need not be described here; typical procedures are described, for example in G.T. Hermanson et al., "Immobilized Affinity Ligand Techniques" (Academic Press, San Diego, 1992), incorporated herein by this reference. Other derivatized constructs can be made with various derivatives for affinity chromatography, according to crosslinking reactions well known in the art. Constructs according to the present invention can also be used in screening for oncogens.

II. <u>CHIMERIC TRANSMEMBRANE PROTEINS</u>

Another approach to the structure and function of transmembrane proteins, particularly integrins, is the use of chimeric transmembrane proteins in which the cytoplasmic region from one transmembrane protein is covalently fused to the extracellular and transmembrane region of another transmembrane protein. Unlike the protein constructs discussed above, this fusion is accomplished by genetic engineering techniques, and each

of the two subunits of the heterodimeric chimeric protein is synthesized by a normal protein synthesis mechanism in which the chain is synthesized as one unit from the amino terminus to the carboxy terminus.

In these chimeric transmembrane proteins, the cytoplasmic domain of an integrin can be fused to the transmembrane and extracellular domains of a non-integrin transmembrane protein, such as the Tac subunit of the human IL-2 receptor. Alternatively, the cytoplasmic domain of one integrin can be fused to the transmembrane and extracellular domains of another integrin.

Examples include a chimeric integrin protein comprising the extracellular and transmembrane domains of the Tac subunit of the IL-2 receptor covalently linked to the cytoplasmic domain of integrin β_3, a chimeric integrin comprising the transmembrane and extracellular domains of the Tac subunit of the IL-2 receptor and the cytoplasmic domain of integrin α_{IIb}.

In the chimeric integrin in which the cytoplasmic domain is that of integrin β_3, the amino acid in the cytoplasmic domain of integrin β_3 that is amino acid 752 in the complete integrin β_3 molecule can be mutated from a serine residue to a proline residue. This mutation is associated with defective inside-out signalling in intact integrins.

Another aspect of the present invention is a heterodimeric chimeric integrin in which the extracellular and transmembrane domains of human integrin $\alpha_{IIb}\beta_3$ are joined to the cytoplasmic domains of human integrin $\alpha_5\beta_1$, so that the α subunit has the extracellular and transmembrane domains of α_{IIb} and the cytoplasmic domain of α_5, and the β subunit has the extracellular and transmembrane domains of β_3 and the cytoplasmic domain of β_1.

Chimeric integrins can be constructed in a number of vectors well known in the art, such as the CDM8 vector (A. Aruffo & B. Seed, <u>Proc. Natl. Acad. Sci. USA</u> 84:8573-

8577 (1987)), or can be constructed in the CMV-IL2R vector for production of chimeric integrins in which the extracellular and transmembrane domains are derived from Tac (S.E. LaFlamme et al., J. Cell Biol. 117:437-447 (1992)). Alternatively, other vectors known in the art can be used.

General cloning techniques are well known in the art and need not be described further here; they are described, for example, in J. Sambrook et al., "Molecular Cloning: A Laboratory Manual" (Cold Harbor Laboratory Press, Cold Spring Harbor, New York, 1989), vol. 1-3; B. Perbal, "A Practical Guide to Molecular Cloning" (John Wiley & Sons, New York, 1988); D.V. Goeddel, ed., "Gene Expression Technology" (Methods in Enzymology, vol. 185, Academic Press, Inc., San Diego, 1991) and other sources. In general, methods for selecting the vector, choosing the appropriate cleavage points for restriction endonucleases, ligating the appropriately cleaved or synthesized segment into the vector, introducing the chimeric vector containing the DNA segment to be expressed into a suitable host cell by techniques such as transfection, lipofection, and other techniques, and expressing the desired protein are well known in the art and described in these references. Therefore, they need not be elaborated upon further here.

Mutations can be introduced into the chimeric integrins by site-specific mutagenic techniques, such as those involving the polymerase chain reaction technique (J.C. Loftus et al., Science 249:915-918 (1990)), as well as other site-specific mutagenic techniques described in the cloning references given above.

The present invention also encompasses nucleic acid sequences encoding chimeric integrins as described above, and nucleic acid sequences operatively linked to at least one control element for transcription of the nucleic acid sequence. Suitable control elements are well known in the art and include promoters and enhancers. These are described in the references cited above, namely Maniotis

et al., Perbal, and Goeddel. Promoters include the Rous sarcoma virus LTR promoter, the SV40 early promoter, the herpes simplex thymidine kinase promoter, the mouse mammary tumor virus LTR promoter, the adenovirus major late promoter, the murine metallothionein promoter, the avian sarcoma virus promoter, the human cytomegalovirus immediate early promoter, the interferon β promoter, the Drosophila heat shock protein 70 promoter and the hMTII promoter. Other promoters and enhancer elements are well known in the art and need not be described further here.

A further aspect of the invention is vectors comprising the nucleic acid sequence coding for a chimeric integrin operatively linked to at least one control element. Vectors according to the present invention are capable of transfecting at least one eukaryotic host for expression of the chimeric integrin encoded by the nucleic acid sequence. Vectors are well known in the art and need not be described further here; they are described in the references cited above. Further examples of vectors are given in the examples.

These chimeric integrins are capable of blocking activation of human integrins. A method for blocking the activation of a human integrin can comprise the step of expressing a chimeric integrin whose extracellular and transmembrane domains are derived from the Tac subunit of the human IL-2 receptor and whose cytoplasmic domain is the cytoplasmic domain of the human β_1 integrin in a quantity sufficient to inhibit high affinity ligand binding by the cellular integrin. This is a competitive method. The preparation of the chimeric integrin whose extracellular and transmembrane domains are derived from the Tac subunit and whose cytoplasmic domain is the cytoplasmic domain of the human β_1 integrin is described in Example 4.

As another aspect of the present invention, a similar method can be used for blocking the activation of a human integrin comprising the step of expressing a chimeric integrin comprising the extracellular

transmembrane domains of the Tac subunit covalently linked to the cytoplasmic domain of integrin β_3.

These techniques block inside-out signal transduction and represents an alternative way of modulating integrin activity other than using small molecular weight competitive inhibitors of ligand binding to the extracellular domain (M.H. Ginsberg et al., J. Biol. Chem. 260:3931-3936 (1985); M.J. Humphries et al. (1986), *supra*). The method described herein involves inhibition of binding to the intracellular domain. Since integrin activation involves cell-type specific factors, such inhibition could be cell type-specific. This provides a totally new method for controlling integrin behavior in disease states such as thrombosis, inflammation, and tumor invasion and metastasis.

The present invention is illustrated by the following Examples. The Examples are for illustrative purposes only and are not intended to limit the invention.

EXAMPLES

Example 1

Synthesis of Type I Protein Construct and Other Model Peptides

The Type I protein construct shown schematically in Figure 1B and in Figure 2 was synthesized, along with the following molecules used as controls: (1) a molecule that consists of the cytoplasmic domain of integrin α_{IIb} with the carboxyl terminal amino acid changed from glutamic acid to glutamine to reflect likely *in vivo* amidation and the cytoplasmic domain of integrin β_3 covalently joined through a thioether linkage, but without the helical segments (MP-2 in Figure 2); (2) a molecule consisting of a helix dimer covalently joined through a thioether linkage without the cytoplasmic domains (Helix-Dimer in Figure 2). The Type I protein construct is identified in Figure 2 and in other figures as MP-1 as an alternative designation.

Also, the cytoplasmic tails of α_{IIb} and β_3 were

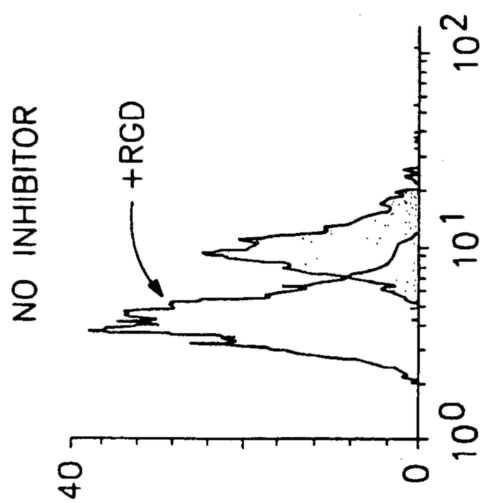


FIG. 10A

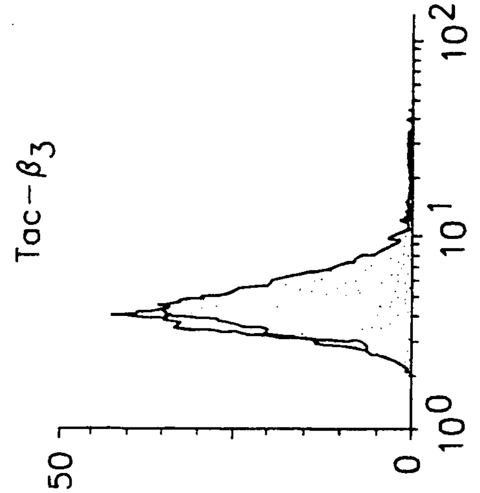


FIG. 10B

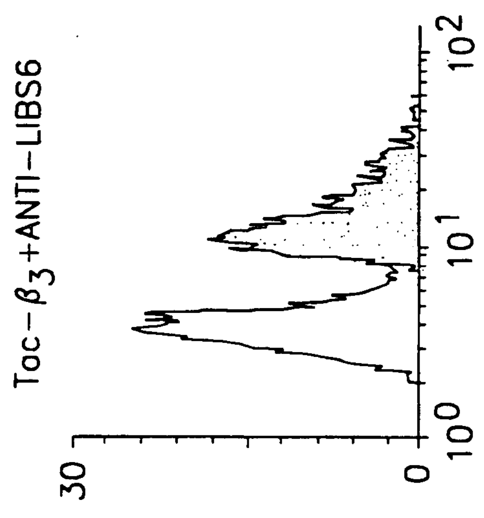


FIG. 10C

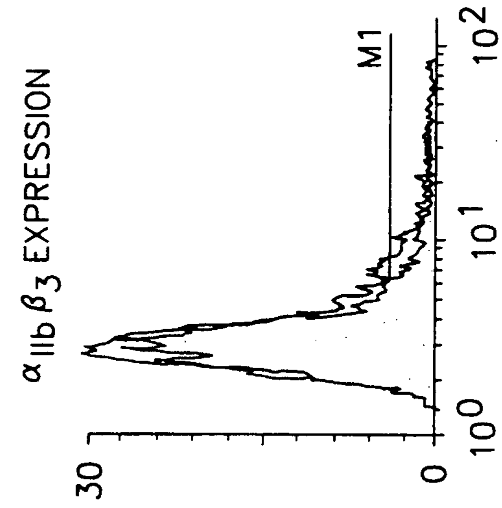
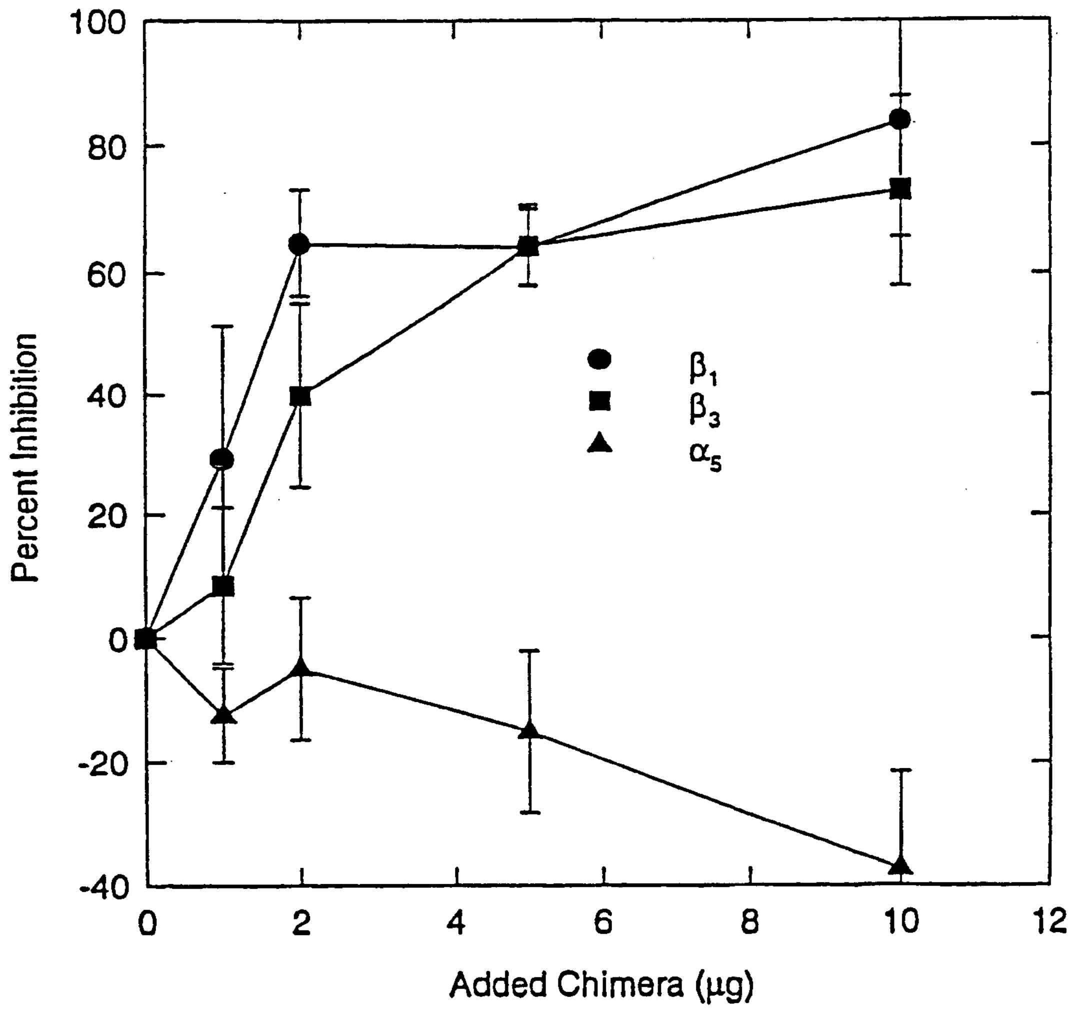


FIG. 10D



100
80
60
40
20
0
-20
-40
Percent Inhibition
0
2
4
6
8
10
12
Added Chimera (μg)
β1
β3
α5

FIG. IIA

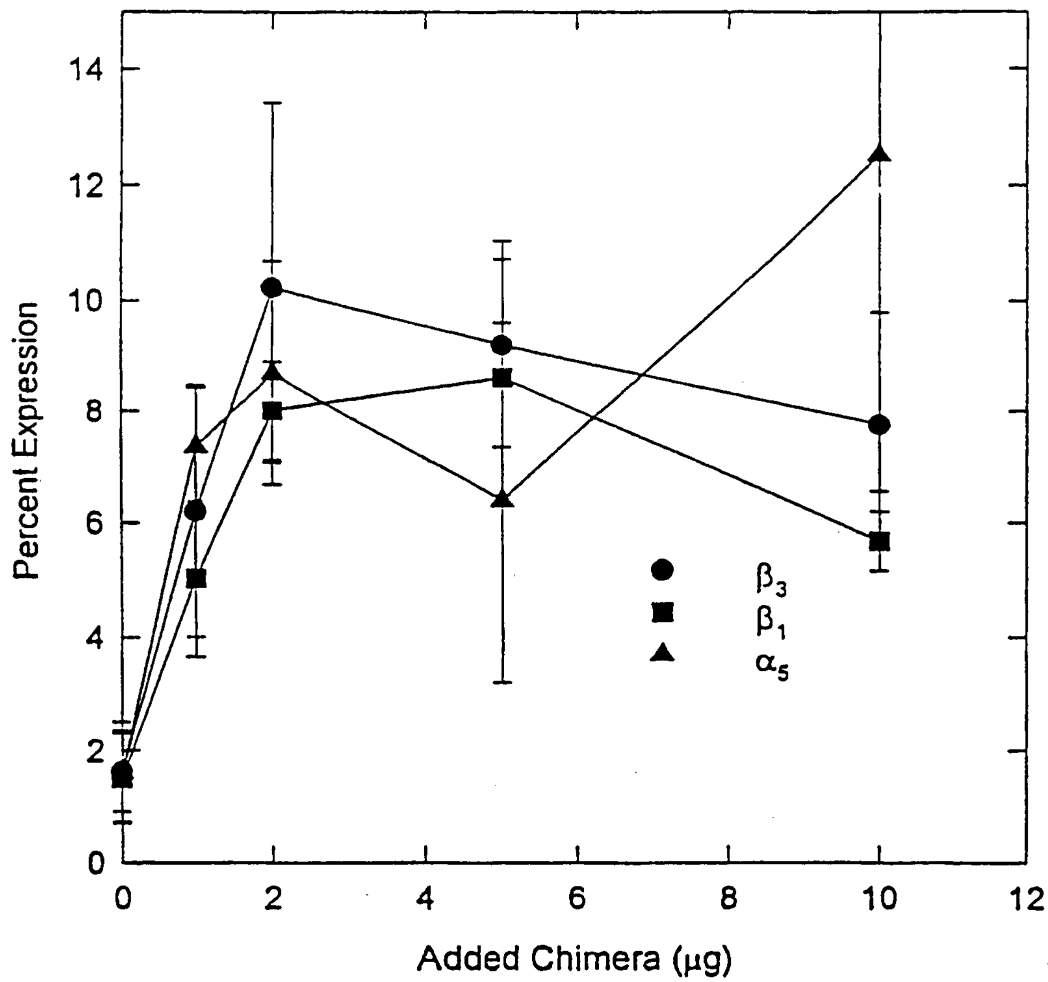

Percent Expression
0
2
4
6
8
10
12
14
Added Chimera (μg)
0
2
4
6
8
10
12
β3
β1
α5

FIG. 11B

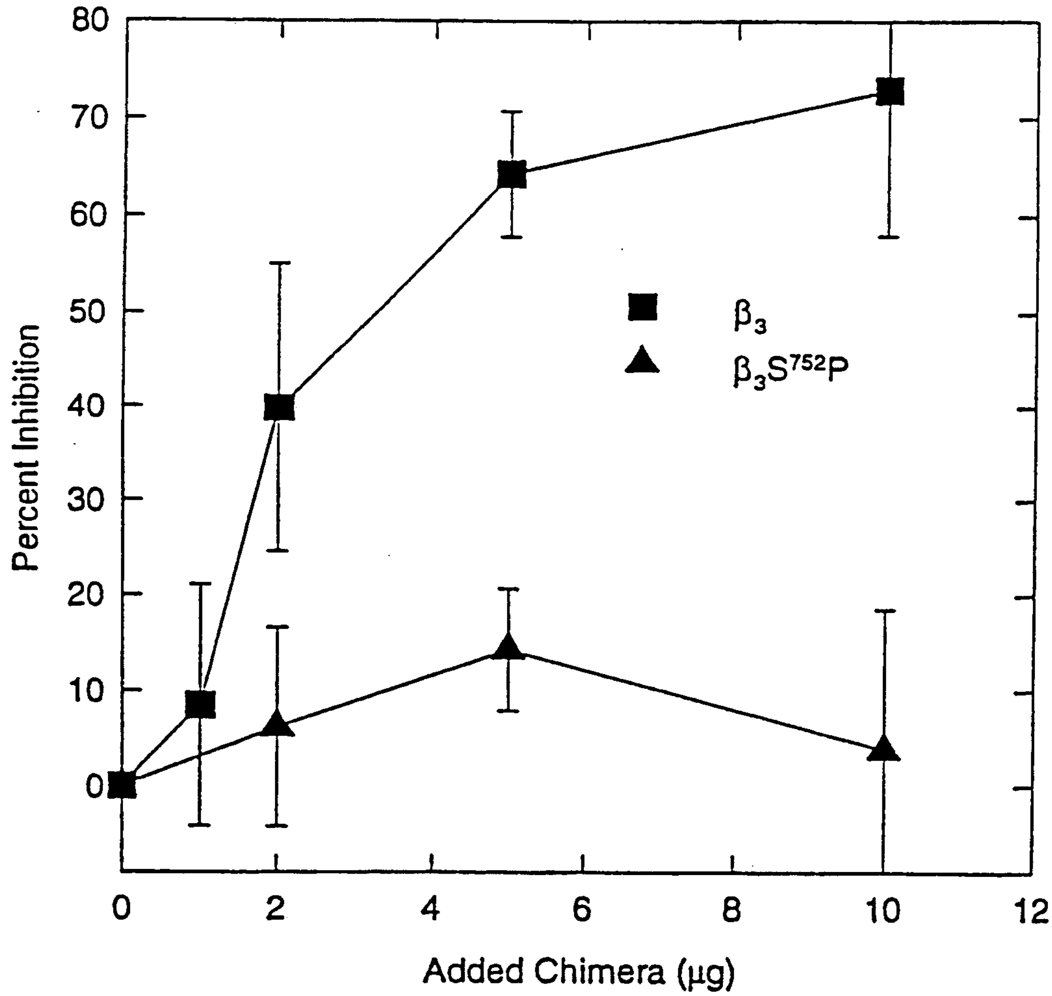

80
70
60
50
40
30
20
10
0
Percent Inhibition
0
2
4
6
8
10
12
Added Chimera (µg)
β3
β3S752P

FIG. 12A

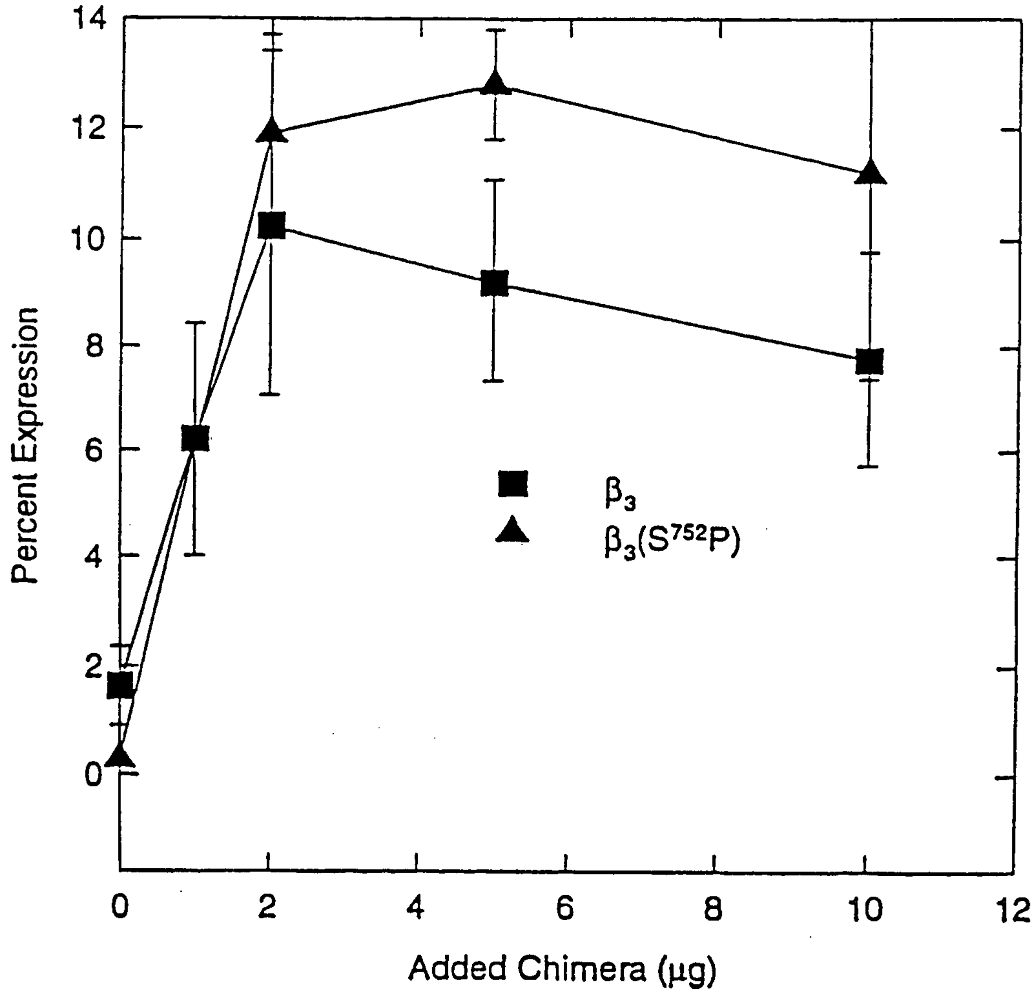

14
12
10
8
6
4
2
0
Percent Expression
0
2
4
6
8
10
12
Added Chimera (µg)
β3
β3(S752P)

FIG. 12B

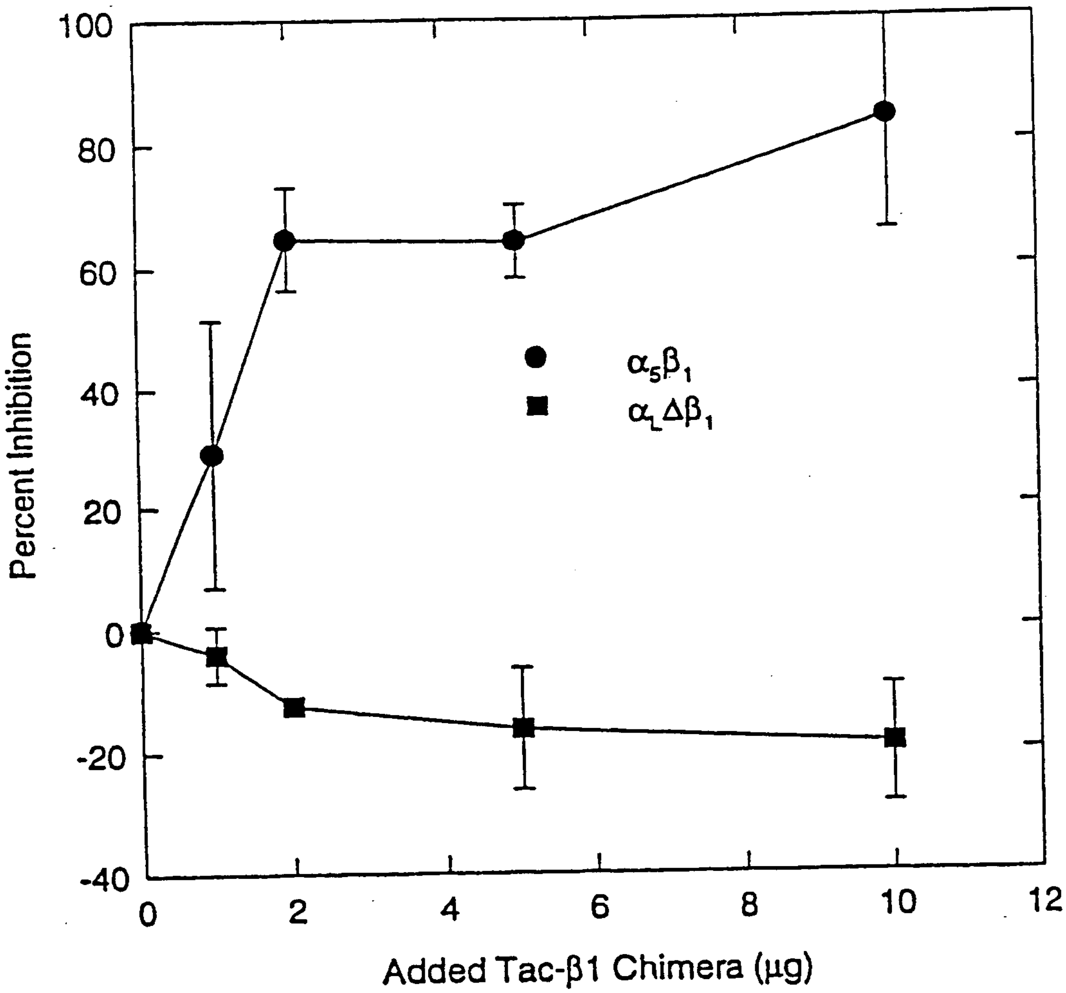

100
80
60
40
20
0
-20
-40
0
2
4
6
8
10
12
Percent Inhibition
Added Tac-β1 Chimera (μg)
$\alpha_5\beta_1$
$\alpha_L\Delta\beta_1$

FIG. 13A

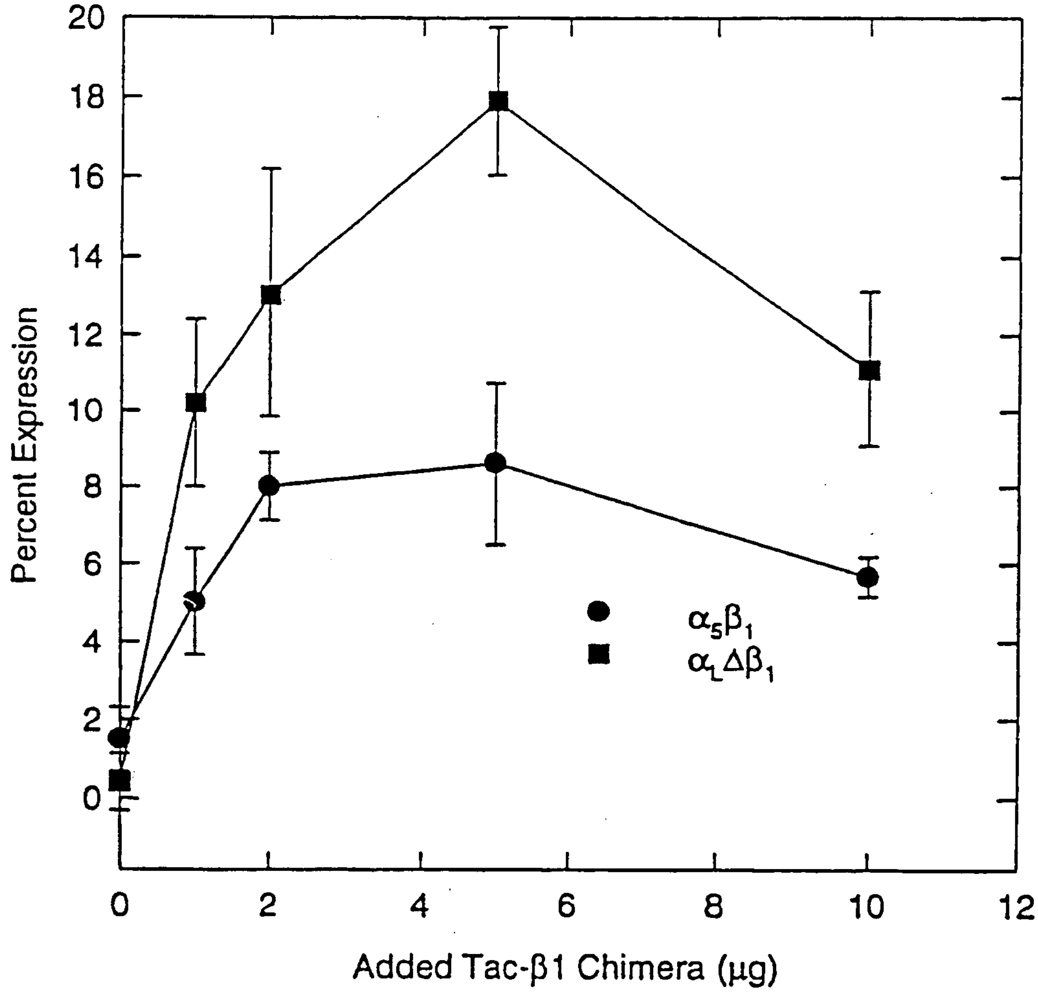

20
18
16
14
12
10
8
6
4
2
0
Percent Expression
0
2
4
6
8
10
12
Added Tac-β1 Chimera (μg)
$\alpha_5\beta_1$
$\alpha_L\Delta\beta_1$

FIG. 13B

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/07542

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12N 5/10, 15/09, 15/12, 15/62, 15/63, 15/79; C07K 19/00, 14/705
US CL :435/69.7, 240.2, 320.1; 514/2; 530/323, 332, 350; 536/23.4, 24.1
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/69.7, 240.2, 320.1; 514/2; 530/323, 332, 350; 536/23.4, 24.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, MEDLINE, BIOSIS
search terms: integrin#, chimer?

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	J. Biol. Chem., Volume 259, Number 21, issued 10 November 1984, Lau et al, "Synthesis of a Model Protein of Defined Secondary and Quaternary Structure," pages 13253-13261, see entire document.	1-32
Y	Science, Volume 256, issued 10 April 1992, Schnolzer and Kent, "Constructing Proteins by Dovetailing Unprotected Synthetic Peptides: Backbone-Engineered HIV Protease," pages 221-225, see pages 222-224 and Figure 2.	1-32
Y	J. Biol. Chem., Volume 268, Number 26, issued 15 September 1993, Monera et al, "Comparison of Antiparallel and Parallel Two-stranded Alpha-helical Coiled-coils," pages 19218-19227, 19219-19221.	1-32

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
07 SEPTEMBER 1995	13 OCT 1995

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	D Freese /for KAREN E. BROWN
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Form PCT/ISA/210 (second sheet)(July 1992)★

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, X ------- P, Y	J. Biol. Chem., Volume 269, Number 28, issued 15 July 1994, Chen et al, "'Inside-out' Signal Transduction Inhibited by Isolated Integrin Cytoplasmic Domains," pages 18307-18310, see pages 18308-18309.	33-47 --------- 1-32
Y	J. Cell Biol., Volume 117, Number 2, issued April 1992, LaFlamme et al, "Regulation of Fibronectin Receptor Distribution," pages 437-447, see pages 439-444.	33-35, 37-45
Y	J. Cell Biol., Volume 114, Number 5, issued September 1991, Solowska et al, "Cytoplasmic and Transmembrane Domains of Integrin Beta 1 and Beta 3 Subunits are Functionally Interchangeable," pages 1079-1088, see pages 1081-1084.	33-35, 37-47
P, X	J. Cell Biol., Volume 126, Number 5, issued September 1994, LaFlamme et al, "Single Subunit Chimeric Integrins as Mimics and Inhibitors of Endogenous Integrin Functions in Receptor Localization, Cell Spreading and Migration, and Matrix Assembly," pages 1287-1298, see pages 1292-1295.	33-35, 37-47
X	J. Biol. Chem., Volume 269, Number 23, issued 10 June 1994, Akiyama et al, "Transmembrane Signal Transduction by Integrin Cytoplasmic Domains Expressed in Single-subunit Chimeras," pages 15961-15964, see entire document.	33-35, 37-47
Y	J. Cell Biol., Volume 125, Number 2, issued April 1994, Pasqualini and Hemler, "Contrasting Roles for Integrin Beta 1 and Beta 5 Cytoplasmic Domains in Subcellular Localization, Cell Proliferation, and Cell Migration," pages 447-460, see pages 449-451.	36
Y	Cell, Volume 68, issued 20 March 1992, Chan et al, "Distinct Cellular Functions Mediated by Different VLA Integrin Alpha Subunit Cytoplasmic Domains," pages 1051-1060, see pages 1052-1055.	36
X	J. Cell Biol., Volume 124, issued March 1994, O'Toole et al, "Integrin Cytoplasmic Domains Mediate Inside-out Signal Transduction," pages 1047-1059, see pages 1050-1051.	36

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/07542

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest ☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet(1))(July 1992)★

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

Group I, claim(s) 1-32, drawn to synthetic protein constructs and methods of making said constructs.

Group II, claim(s) 33-35 and 37-47, drawn to IL-2 receptor-integrin chimeras, a method of making said chimeras and a method of using said chimeras.

Group III, claim(s) 36, drawn to a heterodimeric chimeric integrin.

The inventions listed as Groups I-III do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

The synthetic protein constructs of Group I do not share structural or functional features with the integrin chimeras of Groups II and III, and thus Group I is defined by special technical features which distinguishes it from Groups II and III.

The IL-2 receptor-integrin chimeras of Group II are materially and functionally different and distinct compositions than the heterodimeric chimeric integrin of Group III, and so Groups II and III are defined from one another by special technical and functional features which distinguishes one from the other.

The methods of Group I do not share any special technical features with the methods of Group II because their different process steps are technical features which distinguish them.

Accordingly, the claims are not so linked by a special technical feature within the meaning of PCT Rule 13.2 so as to form a single inventive concept.